NanoAmp™ RT-IVT Labeling Kit

For safety and biohazard guidelines, refer to the "Safety" section in the *NanoAmp™ RT-IVT Labeling Kit Protocol* (PN 4365710). For all chemicals in **bold** type below, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
For materials list, refer to the *NanoAmp™ RT-IVT Labeling Kit Protocol.*
Keep all enzyme components on ice at all times throughout the procedure.

Overview

The purpose of the NanoAmp™ RT-IVT Labeling Kit (PN 4365715) is to convert RNA into digoxigenin (DIG)-labeled cRNA for hybridization to Applied Biosystems microarrays. You have the option of performing a standard one-round amplification or two rounds of amplification.

Workflow

Reverse Transcription

Use T7-oligo(dT) primer. If doing two rounds of amplification, dilute the control RNA 1:10.

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Second Strand Synthesis

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cDNA Purification

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In Vitro Transcription Labeling

If doing a two rounds of amplification, omit Dig-UTP and IVT control DNA

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cRNA Purification

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Purified DIG-labeled cRNA Target

If doing a second-round amplification, this is unlabeled cRNA

Second Round Amplification (Optional)

Second Round Reverse Transcription

Use second round primers

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Second Strand Synthesis

Use T7-oligo(dT) primer

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cDNA Purification

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In Vitro Transcription Labeling

Use Dig-UTP and IVT control DNA

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cRNA Purification

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Purified DIG-labeled cRNA Target

Performing Reverse Transcription

Materials Needed

- Up to 2 µg total RNA for a single round of amplification.
- Reverse transcription reagents (blue-capped tubes)
  - **10× 1st Strand Buffer**
  - Control RNA
  - **RT Enzyme**
  - **RNase Inhibitor**
  - T7-Oligo (dT) primer
  - dNTP Mix
- Nuclease-free water
- 0.2-mL MicroAmp® reaction tube(s)
- Ice bucket
- Pipettor: 1- to 20-µL range
- Pipette tips
- Vortexer
- Microcentrifuge
- Thermal cycler

About Performing Two Rounds of Amplification

IMPORTANT! If you plan to conduct two rounds of amplification, *make the following adjustments to the first round amplification procedure*.

- Before beginning the reverse transcription reaction, make an additional 1:10 dilution of Control RNA in nuclease-free water (for a final dilution of 1:50,000).
- For the first-round IVT reaction ("Performing In Vitro Transcription (IVT) Labeling" on page 3):
  - Replace the Dig-UTP with 8 µL nuclease-free water.
  - Replace the IVT control DNA with nuclease-free water.

  Note: Dig-UTP and IVT control DNA are added to the IVT reaction during the second-round amplification.

Procedure

1. Dilute the control RNA 1:5000.
   a. Add 2 µL control RNA to 98 µL of nuclease-free water and mix by vortexing (1:50).
   b. Add 2 µL of the first dilution to 198 µL of nuclease-free water and mix by vortexing (1:100, final is 1:5000).
2. Thaw sample and reagents on ice.
3. Mix sample and reagents by vortexing and then centrifuge the tubes briefly.
4. Pipette components into 0.2-mL MicroAmp reaction tube on ice:

Component	Volume (µL)
T7-Oligo (dT) primer	1.0
Diluted control RNA (1:5000) (For two rounds of amplification: use an additional 1:10 dilution)	2.0
RNA sample (up to 2 µg total RNA) and nuclease-free water (For two rounds of amplification: use up to 100 ng total RNA)	9.0
Total	12.0

5. Heat and cool the RNA and primer mixture in a thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	70 °C	5 minutes	12 µL
2	4 °C	Indefinite hold	

6. After the run, place the tube on ice.
7. Add components in the following order to the reaction tube and mix thoroughly by pipetting:

   Note: If there is precipitate present in the 10× 1st Strand Buffer, warm it at 37 °C for 5 minutes, then vortex briefly.

Component	Volume (µL)
T7 Oligo Primer, control, and RNA	12.0
10× 1st Strand Buffer	2.0
dNTP Mix	4.0
RT Enzyme	1.0
RNase Inhibitor	1.0
Total	20.0

8. Perform reverse transcription in a thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	25 °C	10 minutes	20 µL
2	42 °C	2 hours	
3	70 °C	5 minutes	
4	4 °C	Indefinite hold	

9. After the run, place the tube on ice.

Performing Second Strand Synthesis

Materials Needed

- 2nd strand synthesis reagents (yellow-capped tubes; dNTPs in blue-capped tubes)
  - 10× 2nd Strand Buffer
  - dNTP Mix
  - **DNA Polymerase**
  - **RNase H**
- Nuclease-free water
- Ice bucket
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw 2nd Strand reagents.
2. Mix the reagents by vortexing and then briefly centrifuge the tubes.

   IMPORTANT! Do not vortex the enzymes.

3. Add components to the cDNA mixture on ice and mix gently:

Component	Volume (µL)
cDNA	20.0
Nuclease-free water	63.0
10× 2nd Strand Buffer	10.0
dNTP Mix	4.0
DNA Polymerase	2.0
RNase H	1.0
Total	100.0

4. Perform second strand synthesis in a thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	16 °C	2 hours	100 µL
2	70 °C	5 minutes	
3	4 °C	Indefinite hold	

5. After the run, place the tube on ice.

Purifying cDNA

Materials Needed

- **DNA Binding Buffer**
- **Wash Buffer**
- Nuclease-free water
- **100% ethanol**, ACS grade or better
- DNA purification column(s)
- DNA elution tube(s)
- Pipettors: 20- to 200-µL range, 100- to 1000-µL range
- Pipette tips
- Microcentrifuge

IMPORTANT! Before you use the kit for the first time, reconstitute the wash buffer by adding 16 mL of 100% ethanol to the Wash Buffer bottle. Mix well and mark the label to indicate that ethanol was added.

Procedure

1. In a new 1.5-mL nuclease-free microcentrifuge tube, combine and mix thoroughly by pipetting up and down:

   Note: If there is precipitate in the DNA Binding Buffer, warm it at 37 °C for 5 minutes, then vortex to mix.

   - **DNA Binding Buffer**: 250 µL
   - Entire 2nd strand synthesis reaction: 100 µL

   IMPORTANT! Sterile, user-supplied tubes should be used here. Do not use the DNA elution tubes in the kit.

2. Begin DNA purification:

   Note: Before using the columns, check each one to be sure the filters are flush against the bottom of the column. If necessary, push them into place with the wide end of a pipet tip.

   a. Pipet the reaction-DNA Binding Buffer mixture (350 µL) to the DNA purification column.
   b. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
   c. Make sure that the entire volume passed through the column. If it did not, centrifuge the column and tube at 10,000 × *g* for 1 minute.
   d. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.

3. Wash the cDNA:
   a. Add 500 µL of Wash Buffer containing ethanol to the column.
   b. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
4. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
5. Elute the cDNA (elution #1):
   a. Transfer the column to a DNA elution tube (provided with the kit).
   b. Pipette 10 µL of nuclease-free water onto the center of the matrix at the bottom of the column.
   c. Incubate the column at room temperature for 2 minutes.
   d. Centrifuge the column and tube at 10,000 × *g* for 1 minute for an elution volume of approximately 9 µL.
6. Elute the cDNA (elution #2):
   a. Pipette 10 µL of nuclease-free water onto the center of the matrix at the bottom of the column.
   b. Incubate the column at room temperature for 2 minutes.
   c. Centrifuge the column and tube at 10,000 × *g* for 1 minute for an elution volume of approximately 18 µL.
7. Discard the column, then close the tube.

   Note: You can store the purified cDNA at –20 °C overnight.

Performing *In Vitro* Transcription (IVT) Labeling

Materials Needed

- IVT labeling reagents (green-capped tubes)
  - **10× IVT Buffer**
  - **NTP Mix**
  - IVT Control DNA
  - IVT Enzyme Mix
- Dig-UTP
- Nuclease-free water
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw the IVT reagents at room temperature.
2. Mix the reagents by vortexing, then briefly centrifuge the tubes.

   IMPORTANT! Do not vortex the enzymes.

   Note: If precipitates are present in the 10× IVT Buffer, warm the buffer at 37 °C for 5 minutes, then briefly vortex it before use.

3. Add the IVT components to the cDNA output at room temperature. Mix gently, then briefly centrifuge the tubes.

Component	Volume (µL)
ds cDNA output – bring volume up to 18 µL with nuclease-free water (If doing two rounds of amplification, bring the volume to 28 µL with nuclease-free water.)	18.0 (28)
10× IVT Buffer	4.0
DIG-UTP (Omit if doing two rounds of amplification.)	8.0 (—)
NTP Mix	4.0
IVT Control DNA (Omit if doing two rounds of amplification.)	2.0 (—)
IVT Enzyme Mix	4.0
Total	40.0

4. Perform IVT in the thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	37 °C	9 hours	40 µL
2	4 °C	Indefinite hold	

5. After the run, remove the tube from the thermal cycler.

Purifying cRNA

Materials Needed

- **RNA Binding Buffer**
- **100% ethanol**
- **Wash Buffer**
- Nuclease-free water
- Ice bucket
- 1.5-mL nuclease-free microcentrifuge tube(s)
- RNA purification column(s)
- RNA collection tube(s) provided in the kit
- Pipettors: 20- to 200-µL range, 100- to 1000-µL range
- Pipette tips
- Microcentrifuge

Procedure

1. In a new 1.5-mL nuclease-free microcentrifuge tube, combine and then vortex briefly to mix:
   - Nuclease-free water: 60 µL
   - Entire IVT reaction: 40 µL

   IMPORTANT! Sterile, user-supplied tubes should be used here. Do not use the RNA collection tubes in the kit.

2. Add to the IVT reaction mix and mix by pipetting (do not vortex):
   - **RNA Binding Buffer**: 350 µL
   - **100% ethanol**: 250 µL
3. Begin the cRNA purification:
   a. Insert an RNA purification column into a RNA collection tube.
   b. Transfer the IVT reaction-RNA Binding Buffer-ethanol mixture (700 µL) to the column, then close the tube.
   c. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
   d. Make sure that the entire volume passed through the column. If it did not, centrifuge the column and tube at 10,000 × *g* for 1 minute.
   e. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
4. Wash the cRNA:
   a. Add 650 µL of **Wash Buffer** to the column, then close the tube.
   b. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
5. Close the tube, then centrifuge the column and tube at 10,000 × *g* for 1 minute.
6. Elute the cRNA:
   a. Transfer the column to a new RNA collection tube (provided with the kit).
   b. Pipette 100 µL of nuclease-free water onto the fiber matrix at the bottom of the column, then close the tube.
   c. Incubate the column at room temperature for 2 minutes.
   d. Centrifuge the column and tube at 10,000 × *g* for 1 minute.
7. Discard the column, then close the tube containing the eluted RNA.
8. Store the cRNA product on ice while you assess the quantity and quality.

Assessing Quantity and Quality

Materials Needed

- TE buffer (10 mM Tris, 1mM EDTA, pH 8.0), user supplied
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Cuvette
- UV spectrophotometer
- 1 to 2% agarose gel

Procedure

1. Measure ultraviolet absorbance:
   a. Dilute a small amount of cRNA product 1:30 with TE buffer.
   b. Measure the absorbance at 260 nm.
2. Calculate concentration and yield:
   - cRNA concentration (µg/µL) = A_{260} × 0.04 µg/µL x 30 (dilution factor)
   - cRNA yield (µg) = cRNA conc (µg/µL) × 100 µL total volume of cRNA purified
3. Prepare a 1 to 2% agarose gel and run between 0.5 and 1 µg of cRNA to visualize the cRNA product.

   Or, analyze an aliquot of cRNA with the Agilent 2100 Bioanalyzer.

Note: The RiboGreen® Quantitation Assay and Kit is optional.

IMPORTANT! When performing the double IVT labeling protocol, accurate quantitation of the 1st round material is essential for the 2nd round amplification and the best assay results.

Concentrating the Purified cRNA (Optional)

If necessary, you can concentrate the cRNA by vacuum centrifugation. Use only the medium or low temperature settings during the drying process. Remove the sample when the desired volume is achieved.

Storing cRNA Product

- –15 to –25 °C for up to 2 months
- –80 °C for long-term storage

Second Round of Amplification (Optional)

Performing the Second Round Reverse Transcription Reaction

1. Prepare 1st Round unlabeled cRNA.

   Using up to 0.1 µg (do not exceed 0.1 µg) of purified aRNA, add nuclease-free water to bring to 10 µL.
2. In a 0.2-mL MicroAmp reaction tube on ice, combine:

Component	Volume (µL)
2nd Round Primer	2.0
1st Round cRNA (up to 0.1 µg) (Adjust volume using nuclease-free water)	10.0
Total	12.0

3. In a thermal cycler with a heated lid, heat and cool the mixture:

Stage	Temperature	Time
1	70 °C	5 minutes
2	4 °C	Hold

4. Place the tube on ice and add:

Component	Volume (µL)
2nd Round Primer and 1st Round cDNA	12
10× 1st Strand Buffer	2.0
dNTP Mix	4.0
RT Enzyme	1.0
RNase Inhibitor	1.0
Total	20.0

5. Mix thoroughly.
6. Run the reverse transcription using the following thermal cycler program:

Stage	Temperature	Time
1	25 °C	10 minutes
2	42 °C	2 hours
3	70 °C	5 minutes
4	4 °C	Hold

Performing the Second Round Second Strand Synthesis

1. Add 1 µL **RNase H** to the tube.

Component	Volume (µL)
RNase H	1.0
cDNA	20.0
Total	21.0

2. Run the following thermal cycler program:

Stage	Temperature	Time
1	37 °C	30 minutes
2	4 °C	Hold

3. Add 5 µL T7-dT_{24} primer.

Component	Volume (µL)
T7-dT_{24} primer	5.0
cDNA	21.0
Total	26.0

4. In a thermal cycler with a heated lid, heat and cool the mixture as follows:

Stage	Temperature	Time
1	70 °C	5 minutes
2	4 °C	Hold

5. Place the tube on ice and add:

Component	Volume (µL)
T7-dT_{24} primer and cDNA	26.0
Nuclease-free water	58.0
10× 2nd Strand Buffer	10.0
dNTP Mix	4.0
DNA Polymerase	2.0
Total	100.0

6. Mix thoroughly.
7. Perform second strand synthesis using the following program in a thermal cycler:

Stage	Temperature	Time
1	16 °C	2 hours
2	70 °C	5 minutes
3	4 °C	Hold

8. Proceed to "Purifying cDNA" on page 3, then proceed to "Performing the Second Round In Vitro Transcription (IVT) Labeling" on page 6.

Performing the Second Round In Vitro Transcription (IVT) Labeling

1. Add the IVT components to the cDNA output at room temperature. Mix gently, then briefly centrifuge.

   Note: If precipitate is present in the 10× IVT Buffer, warm the buffer at 37 °C for 5 minutes, then briefly vortex before use.

Component	Volume (µL)
ds cDNA output – bring volume up to 18 µL with nuclease-free water	18.0
10× IVT Buffer	4.0
DIG-UTP	8.0
NTP Mix	4.0
IVT Control DNA	2.0
IVT Enzyme Mix	4.0
Total	40.0

2. Perform IVT in the thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	37 °C	9 hours	40 µL
2	4 °C	Indefinite hold	

3. Proceed to "Purifying cRNA" on page 4 and follow the remainder of the procedure without further adjustments.

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